



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/GB97/01735 <b>(22) International Filing Date:</b> 30 June 1997 (30.06.97)  <b>(30) Priority Data:</b> 9613754.2 29 June 1996 (29.06.96) GB 9704018.2 26 February 1997 (26.02.97) GB  <b>(71) Applicant (for all designated States except US):</b> THE SCOT- TISH AGRICULTURAL COLLEGE [GB/GB]; West Mains Road, Edinburgh EH9 3JG (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> SURAI, Peter [UA/GB]; Biochemical Sciences Dept., The Scottish Agricultural Col- lege, Auchincruive, Ayr KA6 5HW (GB). NOBLE, Ray- mond, Clifford [GB/GB]; 1 Park Circus, Ayr KA7 2DH (GB).  <b>(74) Agent:</b> MURGITROYD & COMPANY; 373 Scotland Street, Glasgow G5 8QA (GB).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
<b>(54) Title:</b> IMPROVEMENT OF MALE FERTILITY WITH ANTIOXIDANTS AND/OR POLYUNSATURATED FATTY ACIDS		
<b>(57) Abstract</b>  A method of controlling the viability of sperm is disclosed, the method comprising controlling the levels of antioxidants (and/or polyunsaturated fatty acids) in sperm or seminal fluid, or in the diet of the animal producing the sperm.		

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1 IMPROVEMENT OF MALE FERTILITY WITH ANTIOXIDANTS AND/OR POLYUNSATURATED  
2 FATTY ACIDS

3 This invention relates to improvement of male  
4 fertility.

5  
6 All animal species' spermatozoa have high  
7 concentrations of polyunsaturated phospholipids. In  
8 mammalian species e.g. the bull, boar, ram and man, the  
9 substantial level of polyunsaturates present is  
10 characteristically dominated by docosahexaenoic acid  
11 (22:6, n-3), a fatty acid of 22 carbon atoms in chain  
12 length, containing 6 double bonds in n-3 conformation  
13 and belonging to the alpha-linolenic acid (18:3, n-3)  
14 series. Thus in the case of the bovine,  
15 docosahexaenoic acid accounts for around 55% of the  
16 total phospholipid fatty acids, with particular  
17 concentrations occurring within the phosphatidyl  
18 ethanolamine and phosphatidyl choline species. By  
19 contrast, avian spermatozoa exhibit in general very low  
20 concentrations of docosahexaenoic acid and acids of the  
21 n-3 series but this is compensated for by the presence  
22 of substantial concentrations within the phospholipids  
23 of polyunsaturated fatty acids having chain lengths of  
24 20 and 22 carbon atoms, containing 4 double bonds in n-  
25 6 conformation and belonging to th linoleic acid

1 (18:2, n-6) series; these are arachidonic (20:4, n-6)  
2 and docosatetraenoic acid (22:4, n-6) respectively.  
3

4 The lipid composition of the spermatozoan membrane may  
5 be a major determinant of motility, cold sensitivity  
6 and a wide selection of factors associated with overall  
7 viability within fresh ejaculates or stored ejaculates  
8 maintained at -196°C for artificial insemination.  
9

10 According to the present invention there is provided an  
11 antioxidant to enhance sperm function and/or viability.  
12

13 Further according to the present invention there is  
14 provided a polyunsaturated fatty acid (PUFA) to enhance  
15 sperm function and/or viability.  
16

17 Still further according to the present invention there  
18 is provided an antioxidant accompanied by a PUFA to  
19 enhance sperm function and/or viability.  
20

21 The antioxidant and/or PUFA may be administered to the  
22 animal producing the sperm, for example in its diet, or  
23 intravenously or intramuscularly, or may be added to  
24 the sperm or to fluid surrounding the sperm.  
25

26 Preferably the antioxidant is selected from vitamins,  
27 plant extracts and carotenoids.  
28

29 Preferably the PUFA is an n-3 fatty acid, for example  
30 docosahexaenoic acid (DHA) or another member of the  
31 alpha-linolenic acid (18:3, n-3) series.  
32

33 In a further aspect, the present invention provides a  
34 method of enhancing sperm function and/or viability,  
35 comprising adding to the semen of an animal  
36 substantially sperm-free seminal fluid containing an

1 antioxidant and/or a PUFA.

2

3 Th seminal fluid is preferably produced from th semen  
4 of another animal which may have been vasectomised or  
5 from whose semen sperm has been removed.

6

7 The mixture of the semen and seminal fluid can then be  
8 stored at low temperature for use in artificial  
9 insemination.

10

11 The semen in this aspect of the invention may already  
12 have been boosted in function or viability by virtue of  
13 the animal having antioxidant and/or PUFA administered  
14 to it. The PUFA is preferably administered to the  
15 animal in an amount of at least 10mg/kg of body weight,  
16 most preferably 10-45 mg/kg.

17

18 The invention also provides a method of enhancing the  
19 function and/or viability of sperm, the method  
20 comprising controlling the PUFA content of the sperm,  
21 preferably the plasma membrane of the sperm, although  
22 the control of PUFA content of the seminal plasma can  
23 also be of benefit. The PUFA content of the plasma  
24 membrane can be controlled eg by adding PUFA or  
25 antioxidant to the sperm directly or administering the  
26 PUFA or the antioxidant to the animal's diet.

27

28 The invention also provides a method of combatting  
29 sperm dysfunction, comprising controlling the PUFA  
30 content of the sperm, preferably the content of the  
31 sperm plasma membrane, eg by exposing the sperm to a  
32 PUFA or an antioxidant.

33

34 Th term "combat" as used herein refers to th  
35 prevention of a condition (ie prophylactic use) as w ll  
36 as treatment of an existing condition to ameliorate

1 that condition or to delay or prevent its further  
2 deterioration.

3  
4 The PUFA can be added direct to the ejaculate, or can  
5 be administered to an animal to enhance the function  
6 and/or viability of sperm from that animal. In such a  
7 case, the PUFA is preferably administered in quantities  
8 of at least 10-45mg/kg body weight. The PUFA can be  
9 provided in substantially pure form or in combination  
10 with a pharmaceutical carrier or excipient, or in  
11 impure form. For example, the PUFA may be provided in  
12 the form of fish oil, or can be extracted from brain  
13 tissue by conventional methods. The PUFA may be  
14 incorporated into the fatty acid pool of the sperm, or  
15 may remain in the seminal fluid in order to exert its  
16 beneficial effects.

17  
18 The PUFA is preferably a C18-C24 fatty acid.

19  
20 The viability can be enhanced by increased mobility,  
21 cold resistance or related factors.

22  
23 Two embodiments of the invention include:

24  
25 (i) the maximisation of male fertility *in vivo* through  
26 the dietary manipulation of the lipid composition  
27 and/or antioxidant capacities of the fresh  
28 ejaculate.

29  
30 (ii) the development of effective antioxidant/lipid  
31 additives for semen diluents and effective carrier  
32 systems for inclusion of the additives into the  
33 sperm membrane in order to ensure sperm viability  
34 *in vitro* and *in vivo* and fertility capacities  
35 after storage.

36

1     **MATERIALS AND METHODS**

2     The investigations involved both avian (cockerel) and  
3     mammalian (bull) species.

4  
5     (i) Avian dietary treatments.

6     (a) Supplementation with alpha-linolenic acid  
7     (18:3 n-3).

8     Two groups, each of 15 male broiler breeders from the  
9     same genetic stock, were purchased from a commercial  
10    breeder supplier. The males were 21 weeks of age at  
11    the beginning of the experiment and 72 weeks old at the  
12    end. The males were housed in single cages in a  
13    controlled environment with a photoperiod of 13 hours  
14    light: 11 hours dark. They were each fed 130g per day  
15    of feed with 12.5% crude protein and 11.5 MJ/kg of ME.  
16    The control diet was supplemented with soyabean oil (6%  
17    w/w of feed) and the 18:3 (n-3) enriched diet was  
18    produced by supplementation with linseed oil (6% w/w of  
19    feed), see Table 1. Regular lipid analysis of feed was  
20    undertaken to establish the lipid and fatty acid  
21    composition. The males were trained for semen  
22    collection from 21 weeks of age and were milked  
23    routinely twice weekly throughout the experimental  
24    period and three times on the weeks 24, 40 and 54  
25    chosen for laboratory analysis. Lipid analysis was  
26    performed on 5 pooled semen samples.

27  
28    (b) Dietary supplementation with docosahexaenoic acid  
29    (22:6 n-3).

30    Two groups, each of 12 male broiler breeders from the  
31    same genetic stock were used. The males were 11 weeks  
32    of age at the beginning of the experiment with semen  
33    being collected at 24 weeks and 38 weeks of age.  
34    Housing, treatment and control diet were as per example  
35    1. The 22:6 (n-3) enriched diet was produced by  
36    supplementation with a 22:6 (n-3) enriched fish oil

1 extract (3% w/w of feed), see Table 1. Semen  
2 collection was as described in (i)(a).  
3

4 A second trial involving dietary supplementation with  
5 22:6 (n-3) was subsequently undertaken. Details of the  
6 diet, housing and general management of the cockerels  
7 were as for the first trial. The 22:6 (n-3) was  
8 delivered by the inclusion in the diet of the fish oil  
9 at a rate of 5% w/w within the feed. Semen collection  
10 was performed as per (i)(a) above with investigations  
11 of chemical and physiological parameters being  
12 undertaken on samples at 24, 40 and 58 weeks of age.  
13 In this experiment there was a further experimental  
14 group in which 22:6(n-3) was accompanied by the  
15 inclusion of 200 mg/kg of  $\alpha$ -tocopherol in the diet.  
16

17 (c) Supplementation with gamma-linolenic acid  
18 (18:3,n-6).

19 Two groups, each of 20 male broiler breeders from the  
20 same genetic stock were used. The males were 21 weeks  
21 of age at the beginning of the experiment. Housing,  
22 treatment and control diet were as per (i)(a) above.  
23 The 18:3(n-6) enriched diet was produced by  
24 supplementation with evening primrose oil (5% w/w of  
25 feed) containing 9% w/w of 18:3 (n-6), see Table 1.  
26 Semen was collected five times a week at 40 weeks of  
27 age. Lipid analyses was performed on 7 individual  
28 samples.  
29

30  
31 **(ii) Bull dietary treatments**

32 Two groups, each of three Holstein/Freisian bulls from  
33 Scottish Livestock Services AI Centre, Perth, Scotland  
34 comprised the main locus of experimentation; in  
35 addition, observations were made on a Belgian Blue  
36 bull, a breed known for its inherently low level of



1 male fertility. Each bull was kept under standard (and  
2 conventional) conditions appropriate to a leading AI  
3 centre. All the bulls were fed twice daily 3kg of a  
4 standard diet delivering 12.5 MJ/kg ME and 15% crude  
5 protein. Following appropriate collection and sampling  
6 of the ejaculates, each bull was then switched to a  
7 diet, based on the standard diet, but which for each  
8 3kg delivered 90g of a fish oil containing 25% 22:6 (n-  
9 3). Thus with the average bull weighing 800kg,  
10 delivery of 22:6 (n-3) was some 45mg per kg body  
11 weight. The bulls were then sampled after an 8 week  
12 period on this diet. The major fatty acid within the  
13 diets are shown in Table 1. Semen was collected by  
14 artificial vagina.

15  
16 (iii) Spermatozoa evaluation. In the case of the  
17 cockerels sperm quality measurements were made at 24,  
18 39 and 54 weeks of age. Pooled semen samples of 3  
19 ejaculates (5 replicates per group) were analysed in  
20 each case. In the case of the bulls, semen was  
21 collected every 2 weeks over the complete period of the  
22 experiment. Within 20 minutes of collection  
23 appropriate semen parameters were measured that  
24 included ejaculate volume, sperm concentration,  
25 acrosomal integrity and motility using microscopic and  
26 Cellsoft Computer Assisted Analysis. Fertility in the  
27 cockerels was assessed by insemination of laying hens  
28 with a fixed dose of semen ( $70 \times 10^6$  cells/ml). Eggs  
29 were collected for 2 weeks for groups (i)(a) and (i)(b)  
30 and 3 weeks for (i)(c) and incubated for 7 days before  
31 candling to record the presence of any embryo. Weekly  
32 fertilities were measured in groups (i)(a) and (i)(b)  
33 and daily for (i)(c).

34  
35 (iv) Preparation of semen for lipid extraction. Semen  
36 was diluted with an equal volume of 0.85% (w/v) sodium

1 chlorid solution and centrifuged at 700g for 20  
2 minutes at 4°C. The upper diluted plasma layer was  
3 transferred to a fresh tube, the wash procedure was  
4 repeated with 1ml of 0.85% (w/v) sodium chloride and  
5 the final cell pellet was re-suspended in 2ml of 0.85%  
6 (w/v) sodium chloride. In order to obtain sufficient  
7 material for analysis from each individual cockerel,  
8 the successive samples obtained during the 3 week  
9 collection period were combined.

10  
11 (v) Lipid analysis. Total lipids were extracted from  
12 the spermatozoa preparations following homogenisation  
13 in a suitable excess of chloroform:methanol (2:1 v/v).  
14 The lipids were fractionated into their major classes  
15 (phospholipid, free cholesterol, triacylglycerol, free  
16 fatty acids and cholesterol ester) by thin layer  
17 chromatography on silica gel G using a solvent system  
18 of hexane:diethyl ether:formic acid (80:20:1 v/v/v).  
19 Following visualisation under UV light after spraying  
20 with 0.1% w/v solution of 2,7-dichlorofluorescein in  
21 methanol, the separated bands were scraped from the  
22 plates. Phospholipid was eluted from the silica by  
23 washing 3 times with 2ml methanol and the other lipid  
24 classes were similarly eluted with diethyl ether. The  
25 esterified lipid fractions were subjected to  
26 transmethylation by refluxing with  
27 methanol:toluene:sulphuric acid (20:10:1 v/v/v) in the  
28 presence of a pentadecanoic acid standard. The  
29 resultant fatty acid methyl esters were analysed by 1µl  
30 injection, via a CP9010 auto sampler (Chrompack,  
31 London, UK), on to a 30m x 0.25mm diameter, 0.25 µm  
32 film thickness Carbowax capillary column (Alltech UK  
33 Ltd., Carnforth) fitted to a Chrompack CP9001  
34 instrument (Chrompack, London, UK). Integration of the  
35 peaks using an 'EZ-Chrom' Data Handling System (Speck  
36 Analytical, Alloa, UK) enabled the derivation of the

1 fatty acid composition (% w/w of total fatty acids).  
2 The amount of each lipid class was calculated by  
3 comparison of the total fatty acid peak areas to that  
4 of the pentadecanoic fatty acid standard. Free  
5 cholesterol was determined by standard calorimetric  
6 assay (Boehringer, Lewes, UK). Individual phospholipid  
7 classes were separated by high performance thin layer  
8 chromatography (HPTLC) using a solvent system of methyl  
9 acetate: isopropanol:chloroform:methanol:0.25% (w/v in  
10 H<sub>2</sub>O) KCl (25:25:25:10:9 v/v/v/v/v). After charring,  
11 quantification was performed by densitometry using a  
12 Shimadzu CS-9001 PC dual wavelength flying spot thin  
13 layer scanner (Shimadzu Corporation, Japan).  
14

15 (vi) Statistical Analysis. Students t-test was used  
16 for all statistical comparison. Data included 5 + 4  
17 replicates respectively for the cockerels and bulls  
18 included 5 replicates per group at each collection  
19 period for lipid analysis and 15 and 8 replicates per  
20 group at each collection period for semen evaluation.  
21 For cockerels in groups (i)(a) and (i)(b) 5 replicates  
22 within each week of egg collection at each collection  
23 period were used for assessment of fertility and for  
24 cockerels in group (i)(c) 7 replicates were used for  
25 assessment of fertility.  
26

27 (vii) Bull semen diluent  
28 Bulls of known reproductive performance were selected  
29 from Holstein/Friesian and Belgian Blue breeds. Both  
30 groups were known to exhibit problems with routine  
31 freezing of their semen, particularly with respect to  
32 post-freeze survival of spermatozoa and maintenance of  
33 acrosomal integrity. The bulls were 5-6 years of age  
34 and housed/fed according to accepted commercial AI  
35 practice.  
36

1      $\alpha$ -tocopherol additives for addition to fresh diluted  
2     ejaculates were as follows: treatment A, (control) no  
3      $\alpha$ -tocopherol, no semen diluent; treatment B, 10mg/ml  $\alpha$ -  
4     tocopherol, skimmed milk diluent; treatment C, 1mg/ml  
5      $\alpha$ -tocopherol, skimmed milk diluent; treatment D,  
6     10mg/ml  $\alpha$ -tocopherol, egg yolk/biosophus plus diluent;  
7     treatment E, 1mg/ml  $\alpha$ -tocopherol, egg yolk/biosophus  
8     plus diluent; treatment F, 10mg/ml  $\alpha$ -tocopherol, egg  
9     yolk/0.85% (w/v) saline diluent; treatment G, 1mg/ml,  
10    egg yolk/0.85% (w/v) saline diluent.

11  
12    Additive preparation:  $\alpha$ -tocopherol in milk buffer. To  
13    prepare the diluent, 5-50mg of DL- $\alpha$ -tocopherol was  
14    carefully weighed into a fresh test tube. Immediately  
15    afterwards 5 ml of fresh skimmed milk buffer was added  
16    and the preparation mixed thoroughly. To disperse and  
17    ensure complete solubilisation of the vitamin in the  
18    milk the preparation was homogenised thoroughly for  
19    30-40 seconds followed by 10-15 seconds of sonication  
20    until a clean, milky texture was obtained. The  
21    contents of the test tube were then carefully poured  
22    into a darkened glass vial, plugged and stoppered. The  
23    vial was stored immediately at 4°C and out of any  
24    direct sunlight to keep the vitamin and milk in the  
25    best condition for addition to the semen.

26  
27     $\alpha$ -tocopherol in egg yolk. Fresh egg yolk was used to  
28    aid in solubilisation of the lipid-soluble  
29     $\alpha$ -tocopherol. A stock solution of Biosophus Plus 1:4  
30    (v/v) in distilled water was prepared and mixed  
31    thoroughly by manual inversion. A few drops of egg  
32    yolk were placed in the bottom of a clean test-tube and  
33    5-50mg of  $\alpha$ -tocopherol were carefully weighed with the  
34    drops being placed directly onto the egg yolk. The  
35    suspension mixture was then diluted with 5.0ml of the  
36    Biosophus Plus solution and homogenised and stored as

1 described above.

2

3  $\alpha$ -tocopherol in saline buffer. This was prepared as  
4 described above using 5.0ml of physiological saline  
5 (0.85% sodium chloride w/v).  
6

7 In all cases the uniformity of distribution of the  $\alpha$ -  
8 tocopherol throughout the diluent was confirmed before  
9 use by sub-sampling and appropriate analysis based on  
10 high performance liquid chromatography.  
11

12 Semen dilutions. Fresh semen from each bull was placed  
13 in a water bath at 37°C and treated as per routine  
14 semen preparation procedures according to commercial AI  
15 practice. Each ejaculate was divided equally into the  
16 required aliquots for the addition of the additives.  
17 100ul of each additive was added to 1ml of fresh semen.  
18 Semen straws were prepared containing 200ul of semen  
19 plus diluent with a concentration of  $2.5 \times 10^7$   
20 spermatozoa per straw. For each treatment 10 straws  
21 were prepared, half being used for *in vitro* pre-freeze  
22 determinations and the remainder stored at -196°C for  
23 post-freeze determinations 7 days later.  
24

25 Results were obtained from 4 separate collection  
26 periods per bull. Artificial insemination and  
27 assessments of *in vivo* fertility procedures were  
28 performed according to standard AI practices. All  
29 analytical procedures were undertaken as per standard  
30 methodologies.  
31

32 (viii) Avian semen diluent.

33 In the case of the avian a single carrier for the  $\alpha$ -  
34 tocopherol in the semen diluent was assessed. It was  
35 based on the use of seminal plasma harvested from  
36 ejaculates of donor birds of the same breed/stock on

1 which the tests were to be made. The seminal plasma  
2 was harvested by appropriate centrifugation of fresh  
3 semen and in particular, extreme care was taken to  
4 ensure the complete absence of any contaminating cells.  
5

6 To 10mg of  $\alpha$ -tocopherol in an appropriate clean glass  
7 tube was added 5-10ml of the seminal plasma. The whole  
8 was then homogenised for 3-5 minutes followed by  
9 sonication for 1-2 minutes to ensure thorough mixing.  
10 From this stock solution, varying amounts were added to  
11 diluted fresh semen to give a final concentration of  
12 between 10-500ug  $\alpha$ -tocopherol per ml of semen. The  
13 semen was then exposed to combinations of a selection  
14 of storage conditions embracing temperatures of 4° and  
15 37°C and times of 6, 12, 24, 48 and 72 hours.

16 Following exposure the ejaculates were evaluated for *in*  
17 *vivo* fertility and *in vitro* assessment e.g. live sperm  
18 numbers, motility, chemical parameters by standard  
19 microscopic and analytical procedures but to include  
20 also specific tests of sperm viability based on  
21 measurements of membrane integrity by ethidium bromide  
22 and respiration using tetrazolium (reductase activity).  
23 A further test of sperm viability promotion was  
24 undertaken involving the comparison of these  
25 measurements in the presence or absence of  $Fe^{++}$  as a  
26 stimulus for oxidation.  
27

## 28 **Statistical Analysis**

29

30 Students t-test was used for all statistical  
31 comparisons. Analysis of variance and correlations  
32 were undertaken as appropriate.  
33

## 34 **RESULTS**

### 35 **Dietary Supplementation**

1 (i) Cockerel

2 (a) Supplementation with alpha-linolenic acid (18:3 n-  
3 3). The effects of dietary supplementation with 18:3  
4 (n-3) on the characteristics of the semen samples  
5 obtained from cockerels at 24, 40 and 54 weeks of age  
6 are shown in Table 2. For the cockerels on the control  
7 diet, the concentration of spermatozoa in the semen  
8 increased considerably between 24 and 40 weeks of age  
9 and then decreased markedly to 54 weeks. Dietary  
10 supplementation with 18:3 (n-3) significantly increased  
11 spermatozoa concentration at 54 weeks. Also, at 54  
12 weeks the spermatozoa motility was significantly  
13 increased by the supplementation. In the control  
14 cockerels, fertility increased to a maximum at 40 weeks  
15 but had decreased by 54 weeks. The n-3 supplementation  
16 resulted in a significant increase in week 1 fertility  
17 at 40 weeks. Although fertility was not enhanced at 54  
18 weeks, observations of the fertility at 72 weeks (not  
19 shown) were enhanced by n-3 supplementation.

20  
21 The proportions of the major lipid and phospholipid  
22 classes of the spermatozoa are given in Table 3. The  
23 concentration of the total lipid in the spermatozoan  
24 cells increased continually with age; although not  
25 significant these values were higher for the  
26 supplemented birds at 40 and 54 weeks. Phospholipid  
27 was the major lipid class at all stages. However, the  
28 proportion of phospholipid decreased considerably with  
29 age. Supplementation with 18:3 (n-3) did not result  
30 in any dramatic effects on the proportions of the major  
31 lipid classes. Phosphatidyl choline and phosphatidyl  
32 ethanolamine were the main classes of phospholipid but  
33 there were no major effects of n-3 supplementation on  
34 the proportions of the major phospholipid classes.

35

36 The polyunsaturated fatty acid compositions of the

1 total spermatozoan phospholipid from control and n-3  
2 supplemented cockerels are presented in Table 4. The  
3 major polyunsaturated fatty acids in the control  
4 samples were 20:4 (n-6) and 22:4 (n-6); the  
5 phospholipids were almost devoid of n-3 polyunsaturates  
6 apart from the presence of very low levels  
7 (approximately 2% w/w) of 22:6 (n-3). Dietary  
8 supplementation with 18:3 (n-3) resulted in small but  
9 significant effects on these fatty acid profiles. Thus  
10 n-3 supplementation increased the levels of 22:5 (n-3)  
11 at 40 and 54 weeks and 22:6 (n-3) at 54 weeks. Whereas  
12 the levels of 22:6 (n-3) within the phosphatidyl  
13 ethanolamine fraction, normally the major carrier of  
14 the acid, in the control samples at weeks 40 and 54  
15 were negligible, within the treated birds the levels  
16 were 2.2 and 3.1% respectively of total fatty acids  
17 present. However, most notably supplementation  
18 resulted in considerable decreases in the C20-22 n-6:n-  
19 3 ratios at weeks 40 and 54.

20

21 (b) Supplementation with docosahexaenoic acid  
22 (22:6 n-3).

23 Supplementation of the cockerels with 22:6 (n-3)  
24 resulted in an intensive change in overall appearance  
25 and visual parameters of assessment of the ejaculates  
26 at 40 and 58 weeks of age. Sperm concentration  
27 displayed a rise from  $2.08 \times 10^9/\text{ml}$  for the control  
28 group to  $2.23$  and  $2.40 \times 10^9/\text{ml}$  at 40 and 58 weeks of  
29 age respectively for the treated group. A significant  
30 increase in fertility as measured by AI was observed,  
31  $40.5 \pm 6.6(\text{SE})$ ,  $55.4 \pm 4.2$  and  $68.5 \pm 4.9$  respectively.  
32 As can be seen in Tables 5 and 6 the levels of 22:6 (n-  
33 3) within the total phospholipid of the sperm and  
34 throughout all the major individual phospholipid  
35 moieties underwent a significant increase to accompany  
36 this increase in fertility. At the same time ther



1 were extensive and appropriate reductions in total n-  
2 6:n-3 fatty acid ratios. At slaughter at 60 weeks of  
3 age testis (single) weight in the control group was  
4  $15.1\text{g} \pm 1.4$  compared with  $22.3\text{g} \pm 3.0$  for the cockerels  
5 supplemented with 22:6 (n-3) with no change in body  
6 weights.

7  
8 In the second experiment involving 22:6 (n-3)  
9 supplementation, the compositional changes within the  
10 sperm were similar in both absolute and relative terms  
11 to those described for the first experiment (see Tables  
12 5 and 6). Although the inclusion of  $\alpha$ -tocopherol did  
13 not enhance to any significant degree the levels of  
14 polyunsaturates, the content of  $\alpha$ -tocopherol in the  
15 spermatozoa was significantly increased by 60-70% above  
16 the other groups. Inherently the birds used in this  
17 experiment were more fertile (increased sperm number  
18 per unit volume of ejaculate etc.) than for the first  
19 experiments. Again supplementation of the cockerels  
20 with 22:6 (n-3) resulted in extensive changes in  
21 overall appearance and visual parameters of assessment  
22 of the ejaculates at 40 and 58 weeks of age.  
23 Spermatozoa concentration and other major parameters of  
24 fertility are shown in Table 7. As can be seen, semen  
25 volume, total spermatozoa number and fresh and stored  
26 fertilities were all significantly enhanced; relative  
27 spermatozoa motilities were increased by some 15%. The  
28 inclusion of  $\alpha$ -tocopherol had an added effect on  
29 fertility after storage. Testis weights (weights of 2  
30 testes per bird) were again significantly increased by  
31 22:6 (n-3) treatment without any accompanying  
32 differences in body weight. Investigations on the  
33 distribution of 22:6 (n-3) within the spermatozoan cell  
34 showed a preferential incorporation of the acid into  
35 the mitochondria. An important feature arising from  
36 the dietary enhancement with 22:6 (n-3) was an improved

1 fertility of eggs during the 2nd week after artificial  
2 insemination. In approximate terms this equated with  
3 an extra 1.7 eggs becoming available over the whole 2  
4 week period of the fertility investigation compared to  
5 the control treatments.

6

7 **Supplementation with gamma linolenic acid (18:3 n-6)**

8

9 Fatty acid compositions of the major lipid fractions  
10 were unchanged as a result of supplementation with 18:3  
11 n-6. Major spermatozoa features associated with  
12 increased fertility were significantly increased by  
13 18:3 n-6 supplementation (see Table 8); these embraced  
14 motility and fertilities over 1st, 2nd and 3rd weeks  
15 after artificial insemination. Figure 1 shows the  
16 fertility rate on a daily basis following a single  
17 insemination of a fixed dose of  $10 \times 10^7$  spermatozoa.  
18 As can be seen a positive difference in fertilities was  
19 prominent over the 2nd week in particular but also over  
20 the initial part of the 3rd week following insemination  
21 by the 18:3 (n-6) group compared to the control. This  
22 difference equated to an extra 2 fertile eggs per hen  
23 over the 2nd week following artificial insemination.

24

25 **(ii) Bull**

26 As can be seen from a comparison of the various  
27 parameters of sperm evaluation on the 2 diets (see  
28 Table 9), the switch to the diet to which had been  
29 added 22:6 (n-3) had a significant effect across the  
30 board on sperm fertility characteristics.  
31 Appropriately the levels of 22:6 (n-3) within the  
32 phosphatidyl ethanolamine fraction, that is the major  
33 phospholipid moiety associated with 22:6 (n-3),  
34 underwent a significant increase from a pre-treatment  
35 level of  $33.3 \pm 1.0$  (S.E.) to  $60.6 \pm 0.7$  (S.E.) following  
36 treatment ( $p < 0.001$ ). Due to commercial

1 considerations, appropriate fatty acid analysis on the  
2 ejaculat of the single Belgian Blue bull was not  
3 possible.

4  
5 Semen Diluents

6 (i) Bull

7 Determination of  $\alpha$ -tocopherol concentrations within the  
8 semen routinely showed that samples from group A  
9 (control) displayed low levels only of  $\alpha$ -tocopherol, the  
10 levels increasing by some 100 fold with 1mg/ml  $\alpha$ -  
11 tocopherol supplementation and 1000 fold with 10mg/ml  
12  $\alpha$ -tocopherol supplementation. Highest levels of  
13 malondialdehyde within the semen following storage at -  
14 196°C were associated with group A and lowest levels  
15 with 10mg/ml  $\alpha$ -tocopherol supplementation. *In vitro*  
16 parameters of semen quality prior to freezing for the 2  
17 groups of bulls are shown in Tables 10 and 11. The  
18 protocol for commercial semen sale requires a minimum  
19 of 3.5 and 35% for motility and PPM values respectively  
20 for both fresh and frozen semen analysis. Semen  
21 failing to meet such requirements would be discarded.  
22 As can be seen, semen from Group A (control) exhibited  
23 values approximately equal to these minima. By  
24 comparison 3 of the treatments showed a selection of  
25 improvements in motility and greater survival  
26 characteristics. *In vitro* parameters of semen quality  
27 post freezing at -196°C for the 2 groups of bulls are  
28 shown in Tables 12 and 13. Although the quality of the  
29 Belgian Blue semen was not acceptable for commercial  
30 use, improvements were evident as a result of  
31 treatment. Marked improvements in parameters were  
32 exhibited by the Holstein/Fresian bulls.

33  
34 Table 14 gives the results from insemination using  
35 Group B semen samples from the Belgian Blue bulls  
36 following storage at -196°C. As can be seen, in field

1 experim nts th treated semen r sulted in a  
2 considerable enhanc ment of pregnancy.

3

4 Cockerel

5 The inclusion of  $\alpha$ -tocopherol into the diluent, using  
6 harvested seminal plasma as a carrier, significantly  
7 increased resistance of the spermatozoa lipids to  
8 oxidation as based on an extensive range of biochemical  
9 parameters. Thus the level of 22:4, the most  
10 susceptible fatty acid to the lipid oxidation, in the  
11 spermatozoa phospholipids after storage was  
12 significantly higher compared to the control  
13 spermatozoa without vitamin E supplementation (see  
14 Figure 2). The stabilising effect was seen during the  
15 full 24-72 hours of the spermatozoa storage at 4°C.  
16 The increase in storage temperature caused a pronounced  
17 reduction in the level of the long chain  
18 polyunsaturated fatty acids in the phospholipids.  
19 Under such conditions the diluent was also effective as  
20 a protective agent against oxidation (see Figure 3).

21

22 Storage was also associated with reduction of reductase  
23 activity, a feature that reflects damage to the  
24 respiratory chain of mitochondria of the spermatozoa  
25 involving peroxidation of the mitochondria lipids. The  
26 diluent clearly preserved the spermatozoa mitochondria  
27 lipids from oxidation and promoted reductase activity  
28 at both 4°C and 37°C (see Figures 4 and 5  
29 respectively).

30

31 The main problem of spermatozoa storage is membrane  
32 damage as a result of lipid peroxidation. Under such  
33 conditions membrane permeability is dramatically  
34 incr as d and fertilisation capacity reduced. As can  
35 b seen from Figures 6 and 7 the inclusion of the  
36 diluent significantly increased sperm membrane

1 integrity after storage at both 4°C and 37°C. Th  
2 effect of all these parameter chang s under normal  
3 conditions is to diminish considerably sperm motility  
4 after storage. A pronounced protective effect of the  
5 diluent on spermatozoa motility following storage at  
6 4°C and 37°C was observed (see Figure 8 and 9). That  
7 vitamin E was distributed uniformly throughout the  
8 semen was verified by appropriate determination of  $\alpha$ -  
9 tocopherol from different parts of diluted semen (see  
10 Figure 10). It was significant that after incubation  
11 of the spermatozoa, some 8% of the  $\alpha$ -tocopherol had  
12 become incorporated into the membranes (See Figure 11)  
13 and it was not possible to remove it during 3-5  
14 consecutive washings with pure diluent. Confirmation  
15 of the protective effect of the diluent against lipid  
16 peroxidation sperm was further obtained by incubation  
17 of the spermatozoa in the presence of  $Fe^{2+}$  at 37°C.  
18 The results obtained (see Figure 12) indicate that  
19 malondialdehyde accumulation was less than one third  
20 that of the control spermatozoa.

21

## 22 DISCUSSION

23

24 There is an overwhelming preponderance of linoleic  
25 (18:2 n-6) in proprietary feeds of domestic farm  
26 animals. Other fatty acids of the n-6 series and those  
27 of the n-3 series are notable by their virtual absence.  
28 That such a predominance of linoleic acid may not  
29 always be wholly beneficial to the well-being and  
30 health of the animal through effects upon tissue fatty  
31 acid composition and aspects of metabolism is now being  
32 asked. With such a high-profile presence of long chain  
33 polyunsaturated fatty acids of the n-3 series in  
34 mammalian sperm lipids, it is suggested that the  
35 alteration of the current fatty acid profile of animal  
36 proprietary feeds towards increasing levels of acids of

1 the n-3 series may be highly relevant to the ontogeny  
2 of the characteristic fatty acid profiles and  
3 subsequent function of the sperm. Similarly, in the  
4 avian the high profile presence of C20 and C22  
5 polyunsaturates of the n-6 series would also suggest  
6 the need to attempt their improved availability.  
7 Presently reported are the results from experiments  
8 designed to evaluate the deliberate enhancement of the  
9 diets of the cockerel and bull with fatty acids of the  
10 n-3 series and n-6 upon the lipid/fatty acid profile of  
11 the spermatozoa and associated changes to parameters of  
12 spermatozoa function and fertility.

13  
14 It is clear from the analyses that initial lipid/fatty  
15 acid compositions of the spermatozoa of the 2 species  
16 conformed to that which has been previously reported.  
17 Thus, whereas in both species the lipids of the  
18 spermatozoa displayed extremely high levels of  
19 polyunsaturates, in the bull there was predominance of  
20 22:6 (n-3) and in the cockerel 22:4 (n-6). The  
21 apparent substitution of 22:4 (n-6) for 22:6 (n-3) in  
22 the cockerel can be suggested to be the reaction to an  
23 almost complete domination in the diet of linoleic acid  
24 (18:2, n-6) and thereby determining that 22:4 (n-6) as  
25 opposed to 22:6 (n-3) be the long chain polyunsaturate  
26 for spermatozoa inclusion.

27  
28 The inclusion of the n-3 fatty acids in the diet was to  
29 increase significantly their levels within the  
30 spermatozoa and to have extensive beneficial effects on  
31 parameters of spermatozoa function and therefore male  
32 fertility in the species. Although in the case of the  
33 cockerel there was a marked difference in the levels  
34 attainable within the spermatozoa of the long chain n-3  
35 polyunsaturates, nevertheless effects on spermatozoa  
36 parameters were very positive. 22:6 (n-3) is an

1 extensively available fatty acid. It is clear from the  
2 present results that deliberate enhancement of this  
3 acid within the diet of the cockerel and bull and also  
4 long chain polyunsaturates of the n-6 series in the  
5 cockerel presents a simple and effective means of  
6 promoting a range of parameters that lead to increased  
7 spermatozoa quality, output and viability at  
8 ejaculation. In the case of the cockerel, the result  
9 was to lead to a significant increase in output of the  
10 fertile eggs from the hen, a most important feature to  
11 commercial production. Similarly in the case of the  
12 bull, a dramatic decrease in "non return" rates of  
13 heifers was observed.

14  
15 Intensive animal production systems require an  
16 efficient insemination service, both natural and  
17 artificial. This is clearly dependent not only upon  
18 maximising the initial fertility of fresh ejaculates  
19 but also its maintenance during storage. The need  
20 exists to extend the life of semen for a fresh delivery  
21 service and enhance the ability to maintain spermatozoa  
22 function during the following cryoscopic storage in all  
23 farm animal species.

24  
25 The present data have clearly demonstrated the ability  
26 to promote the maintenance of spermatozoa viability and  
27 function following cryoscopic storage through the  
28 addition of  $\alpha$ -tocopherol, in particular through a  
29 unique carrier medium. A very broad range of  
30 spermatozoa characteristics were able to be increased  
31 compared with spermatozoa maintained under standard  
32 cryoscopic AI conditions. Apart from measurements in  
33 vivo, the effect of the carrier/ $\alpha$ -tocopherol medium was  
34 to prevent the significant reductions that arise as a  
35 result of storage in a range of biochemical and  
36 physiological features that are known to be intimately

1 associated with spermatozoa viability and function.  
2 Th data clearly demonstrates a means whereby a  
3 significant enhanc ment of male fertility can be  
4 obtained following sperm storage in the liquid state.  
5

6 The present work therefore underlines 2 major vectors  
7 through which male fertility in mammalian and avian  
8 species may be significantly enhanced with appropriate  
9 and significant benefits to subsequent stock  
10 production:  
11

12 (i) by the deliberate manipulation of the spectrum and  
13 level of long chain fatty acid combinations within  
14 the spermatozoa by appropriate dietary means.  
15

16 (ii) by the addition to the ejaculate prior to  
17 cryoscopic and hypothermic storage of  $\alpha$ -tocopherol  
18 through a unique carrier medium, including  
19 harvested seminal fluid lipids from donor animals.  
20

21 It is clear that the invention is transferable across  
22 species to include the human.  
23



Table 1. The major fatty acids (per cent by weight of total) in the diets.

	Cockerel				Bull	
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 1	Diet 2
<i>major fatty acids :</i>	<i>(soyabean oil)</i>	<i>(linseed oil)</i>	<i>(fish oil)</i>	<i>(evening primrose oil)</i>	<i>(soyabean oil)</i>	<i>(fish oil)</i>
palmitic (16:0)	12	10	22	10	15	20
stearic (18:0)	4	4	6	2	3	4
oleic (18:1, n-9)	23	21	19	13	17	16
linoleic (18:2, n-6)	50	30	20	62	52	32
linolenic (18:3, n-3)	6	34	3	2	6	4
docosahexaenoic (22:6, n-3)	<1	<1	14		<1	11
				5 (18:3n-6)		

Table 2. The effect of linolenic acid (18:3, n-3) supplementation on semen characteristics of the cockerel.

	Week 24		Week 40		Week 54	
	Control	Treated	Control	Treated	Control	Treated
<i>Sperm conc.</i> (10 <sup>9</sup> cells/ml)	4.7 ± 0.5	5.0 ± 0.6	7.7 ± 0.6	7.6 ± 0.6	5.1 ± 0.9	6.8 ± 0.7*
<i>motility (1%)</i>	56.4 ± 4.1	56.5 ± 4.4	54.5 ± 3.8	62.5 ± 5.1	33.8 ± 3.9	53.9 ± 4.7**
<i>fertility (%)</i>						
Week 1	68.3 ± 4.9	62.7 ± 9.1	82.8 ± 4.9	96.8 ± 3.2*	74.4 ± 4.6	76.8 ± 3.8
Week 2	45.0 ± 7.1	58.4 ± 10.8	61.7 ± 4.7	57.5 ± 7.3	47.9 ± 6.4	54.6 ± 8.4

Values are means ± standard error. Significance of difference between control and treated: \* p < 0.05, \*\* p < 0.01

**Table 3. The effect of linolenic acid (18:3 n-3) supplementation on the concentration and proportion of the major lipid and phospholipid classes (per cent by weight of total) in the spermatozoa of the cockerel.**

	Week 24		Week 40		Week 54	
	Control	Treated	Control	Treated	Control	Treated
<i>Total lipid</i> <i>µg/109 cells</i>	261.2 ± 12.1	240.0 ± 27.6	274.4 ± 16.9	316.0 ± 44.0	364.4 ± 75.3	427.7 ± 84.1
<i>Lipid class</i> <i>(% w/w of total lipid)</i>						
PL	60.1 ± 2.3	68.3 ± 4.4	69.2 ± 2.1	68.5 ± 1.6	57.7 ± 1.4	57.4 ± 3.4
FC	12.6 ± 1.4	12.1 ± 2.6	12.9 ± 0.6	17.8 ± 0.9	24.8 ± 1.8	23.8 ± 1.8
FFA	4.9 ± 1.6	5.9 ± 1.0	6.9 ± 1.2	5.3 ± 0.5	9.0 ± 1.8	4.9 ± 1.4
TG	9.3 ± 3.1	5.3 ± 2.8	3.8 ± 1.1	3.2 ± 2.1	3.1 ± 0.7	4.7 ± 1.3
CE	13.1 ± 3.6	8.4 ± 3.9	7.2 ± 1.2	5.2 ± 0.8	8.5 ± 0.6	9.2 ± 2.2
<i>Phospholipid class</i> <i>(% w/w of total phospholipid)</i>						
PC	32.3 ± 1.5	33.4 ± 1.1	25.4 ± 1.6	26.6 ± 1.3	34.7 ± 1.2	32.4 ± 1.1
PE	33.1 ± 1.6	31.7 ± 1.2	33.8 ± 1.2	32.9 ± 0.8	31.1 ± 0.4	32.9 ± 0.9
PS	19.2 ± 2.1	18.9 ± 0.7	24.4 ± 0.7	22.3 ± 0.8	20.9 ± 0.8	21.6 ± 0.5
Sph	10.5 ± 1.1	11.9 ± 1.3	11.5 ± 0.4	12.9 ± 2.9	8.4 ± 0.5	9.4 ± 0.5
CL	4.9 ± 0.5	4.1 ± 0.5	4.8 ± 0.4	5.2 ± 0.8	4.9 ± 0.3	3.7 ± 0.2

Values are means ± standard error.

PL = phospholipid; FC = free cholesterol; FFA = free fatty acid; TG = triacylglycerol; CE = cholesterol ester  
 PC = phosphatidyl choline; PE = phosphatidyl ethanolamine; PS = phosphatidyl serine; Sph = sphingomyelin;  
 CL = cardiolipin.

**Table 4. The effect of linolenic acid (18:3, n-3) supplementation on the polyunsaturated fatty acid concentrations (per cent by weight of total fatty acids) within the phospholipid fraction of the spermatozoa of the cockerel.**

	Week 24		Week 40		Week 54	
	Control	Treated	Control	Treated	Control	Treated
<i>n-6 acids:</i>						
18:2 (n-6)	2.7 ± 0.2	2.4 ± 0.1	3.4 ± 0.5	2.3 ± 0.1	4.7 ± 0.4	3.7 ± 0.3
20:4 (n-6)	12.5 ± 0.4	13.1 ± 0.4	11.7 ± 0.4	12.1 ± 0.5	11.9 ± 0.2	11.7 ± 0.2
22:4 (n-6)	22.8 ± 1.1	23.0 ± 0.8	22.9 ± 1.0	19.9 ± 0.8	21.7 ± 1.4	19.2 ± 0.6
<i>n-3 acids:</i>						
18:3 (n-3)	0.8 ± 0.5	1.2 ± 0.7	nd	0.4 ± 0.03	nd	nd
22:5 (n-3)	1.0 ± 0.2	0.8 ± 0.2	1.0 ± 0.1	5.3 ± 0.9**	0.8 ± 0.05	3.4 ± 0.1**
22:6 (n-3)	2.2 ± 0.1	2.4 ± 0.1	2.5 ± 0.2	2.3 ± 0.1	1.9 ± 0.1	2.4 ± 0.1*
C20-22 n-6/n-3 ratio	10.5 ± 1.4	10.0 ± 1.8	10.9 ± 0.5	4.5 ± 0.6**	14.5 ± 0.6	5.6 ± 0.2**

Values are means ± standard error; nd = not detectable. Significance of difference between control and treated: \*p < 0.05; \*\* p < 0.01.

Table 5. The effect of docosahexaenoic acid (22:6 n-3) supplementation on the C20 and C22 polyunsaturated fatty acid concentrations (per cent by weight of total fatty acids) within the phospholipid fraction of the spermatozoa of the cockerel.

	Week 24		Week 40		Week 58	
	Control	Treated	Control	Treated	Control	Treated
20:4 (n-6)	13.0 ± 0.1	8.9 ± 0.1***	11.4 ± 0.3	8.6 ± 0.2***	10.7 ± 0.2	8.3 ± 0.2***
22:4 (n-6)	19.5 ± 0.8	8.5 ± 0.4***	21.7 ± 0.2	15.0 ± 0.6***	18.2 ± 0.4	12.4 ± 0.5***
22:5 (n-3)	nd	nd	nd	nd	1.9 ± 0.01	3.1 ± 0.1***
22:6 (n-3)	4.7 ± 0.1	13.3 ± 0.5***	3.8 ± 1.1	10.1 ± 0.2***	5.1 ± 0.2	9.1 ± 0.3***
C20-22 n-6/n-3 ratio	6.9	1.3***	8.7	2.3**	4.1	1.7**

Values are means ± standard error. nd = not detectable. Significance of difference between control and treated: \*\*\* p < 0.001.

**Table 6. The effect of docosahexaenoic acid (22:6 n-3) supplementation on the concentration of 22:6 (n-3) (per cent by weight of total fatty acid) within the major phospholipid fractions of the spermatozoa of the cockerel.**

	Week 24		Week 40		Week 58	
	C	T	C	T	C	T
PC	1.7 ± 0.3	7.0 ± 0.3***	1.1 ± 0.3	5.2 ± 0.4***	2.6 ± 0.2	5.7 ± 2.7
PE	6.7 ± 0.7	22.6 ± 1.4***	6.1 ± 1.2	16.4 ± 1.0***	9.4 ± 0.2	14.9 ± 1.1**
PS	6.1 ± 0.9	20.5 ± 2.0***	5.6 ± 0.4	17.5 ± 1.1***	7.1 ± 0.7	13.1 ± 0.7***
Sp1	4.8 ± 2.6	16.4 ± 2.3*	25.5 ± 3.6	11.2 ± 3.6*	14.4 ± 2.2	17.7 ± 2.3
CL	9.2 ± 0.2	24.2 ± 0.7***	5.3 ± 0.8	14.0 ± 1.6**	2.7 ± 0.3	14.3 ± 1.6**

Values are means ± standard error. Significance of difference between control and treated: \*\* p < 0.01; \*\*\* p < 0.001.

PL - phospholipid; FC - free cholesterol; FFA - free fatty acid; TG - triacylglycerol; CE - cholesterol ester

PC - phosphatidyl choline; PE - phosphatidyl ethanolamine; PS - phosphatidyl serine; Sph - sphingomyelin; CL - cardiolipin.

Table 7. The effect of docosahexaenoic acid (22:6 n-3) supplementation on the major cockerel's sperm parameters at 50 weeks of age. The results of the 2nd experiment.

Diet	Control (maize oil)	DHA	DHA + Vit. E
Semen volume, ml	0.25	0.45**	0.40**
Spermatozoa concentration 10 <sup>9</sup> /ml	3.05	3.11	3.01
Total number of spermatozoa, 10 <sup>9</sup> /ejaculate	0.763	1.400**	1.204**
Fertilizing capacity of the fresh semen, %	80.6	84.8*	86.9**
Fertilizing capacity of stored 24 h at 4°C semen %	70.4	69.6	77.9**
Testes weight, g	20.95	30.11**	37.14**
Body weight, kg	5.37	5.69	5.89

Values are means. Significance of differences between control and treated groups. \*/-P < 0.05; \*\*/-P, 0.01

**Table 8. The effect of GLA (18:3n-6) supplementation on the major cockerel's sperm parameters at 40 weeks of age.**

<b>Sperm parameters</b>	<b>Control</b>	<b>GLA</b>
volume ml	0.62 ± 0.04	0.66 ± 0.07
concentration 10 <sup>9</sup> /ml	3.70 ± 0.20	2.74 ± 0.37
total sperm 10 <sup>9</sup> /ejaculate	2.28 ± 0.19	1.84 ± 0.35
motility %	48.9 ± 3.11	54.0 ± 3.70
fertility 1st week <sup>2</sup>	92.8 ± 2.58	90.3 ± 2.15
fertility 2nd week <sup>3</sup>	58.1 ± 5.46	74.9 ± 5.18
fertility 3rd week <sup>4</sup>	13.9 ± 3.32	18.9 ± 5.18

<sup>2</sup> fertility of the 1st week after AI, <sup>3</sup> fertility of the 2nd week after AI, <sup>4</sup> fertility of the 3rd week after AI.



**Table 9. The effect of docosahexaenoic acid (22:6, n-3) supplementation on semen characteristics of the bull.**

	<b>Sperm conc. (10<sup>9</sup> cells/ml)</b>	<b>Standard Motility (%)</b>	<b>Standard PPM (%)</b>	<b>Acrosomal integrity (%)</b>
<i>Freisian/Holstein:</i>				
pre-treatment	0.6 ± 0.1	3.3 ± 0.2	21.7 ± 4.4	70.0 ± 1.2
post-treatment	0.9 ± 0.1*	4.0 ± 0.1*	36.0 ± 0.6*	90.0 ± 2.0***
<i>Belgian Blue:</i>				
pre-treatment	1.27	3.4	25	68
post-treatment	2.82	4.0	36	80

Values are mean ± standard error. Significance of difference between pre and post-treatment: \* p < 0.05;  
 \*\* p < 0.01.

Table 10. Fresh semen *in vitro* characteristics of the Belgian Blue.

treatment	Citrate test		Standard drop	
	motility	PPM	motility	PPM
A	3	28	4	39
B	4*	38*	4	40*
C	3.5	35*	4	38
D	3	30*	4	37
E	3	27	4	35
F	3	15	3.5	34
G	3	18	3.5	36

\* Parameters greater than those of the control

Table 11. Fresh semen *in vitro* characteristics of the Holstein/Friesian.

treatment	Citrate test		Standard drop	
	motility	PPM	motility	PPM
A	3.5	36	3.5	35
B	4*	37*	4*	38*
C	4*	35	4*	36*
D	3	36	3.5	36*
E	3.5	35	3.5	34
F	4*	38*	3.5	35
G	3.5	36	4*	37*

\* Parameters greater than those of the control.

Table 12. Frozen semen *in vitro* characteristics of the Belgian Blue following storage at -196°C.

treatment	Citrate test		Standard drop		Acrosomal integrity %		
	motility	PPM	motility	PPM	abnormal	non intact	intact
A	3	22	3	10	31	28	72
B	3	20	2.5	13*	37	29	71
C	3	15	2.5	13*	33	29	71
D	3.5	15	2	7	30	28	72
E	2	17	2.5	12*	37	39	61
F	2.5	20	3	8	14*	34	66
G	2	25*	3	10	14*	56	44

\*Parameters greater than those of the control.

Table 13. Frozen semen *in vitro* characteristics of the Holstein/Fresian following storage at -196°C.

treatment	Citrate test		Standard drop		Acrosomal integrity %		
	motility	PPM	motility	PPM	abnormal	non intact	intact
A	3.5	34	3	23	16	18	82
B	4*	35*	3.5*	29*	13*	14*	86*
C	3.5	30	3.5*	22	14*	17*	83*
D	3.5	30	3	18	10*	18	82
E	3	25	3	20	16	36	64
F	3	10	2.5	15	10*	28	72
G	3.5	31	3	14	13*	18	82

\*Parameters greater than those of the control.

Table 14. in vivo inseminations performed on synchronised heifers.

Treatment	A	B
<i>Trial 1:</i>		
% heifers pregnant	56	64
<i>Trial 2:</i>		
% heifers pregnant	31	55

1     Claims:

2

3     1   The use of an antioxidant to enhance sperm function  
4     and/or viability.

5

6     2   The use of a polyunsaturated fatty acid (PUFA) to  
7     enhance sperm function and/or viability.

8

9     3   The use of an antioxidant accompanied by a PUFA to  
10    enhance sperm function and/or viability.

11

12    4   The use claimed in any of the preceding Claims,  
13    wherein the antioxidant and/or the PUFA is administered  
14    to the animal producing the sperm.

15

16    5   The use claimed in any of Claim 1 to 3, wherein the  
17    antioxidant and/or the PUFA is added to the sperm or to  
18    fluid surrounding the sperm.

19

20    6   The use claimed in any of Claims 2 to 5, wherein the  
21    PUFA is an n-3 fatty acid.

22

23    7   The use claimed in any Claims 1 and 4 to 6, wherein  
24    the antioxidant is selected from vitamins, plant  
25    extracts and carotenoids.

26

27    8   The use of a PUFA to enhance sperm function and/or  
28    viability in avians, wherein the PUFA is an n-6 fatty  
29    acid.

30

31    9   A method of enhancing sperm function and/or  
32    viability, comprising adding to the semen of an animal  
33    substantially sperm-free seminal fluid containing an  
34    antioxidant and/or a PUFA.

35

36    10   A method as claimed in Claim 9, wherein said

1 seminal fluid is produced from the semen of another  
2 animal.

3

4 11 A method as claimed in Claim 9 or 10, wherein said  
5 seminal fluid is produced by removing sperm from the  
6 semen of an animal.

7

8 12 A method as claimed in Claim 9, 10 or 11, wherein  
9 the antioxidant is selected from vitamins, plant  
10 extracts and carotenoids.

11

12 13 A method as claimed in any of Claims 9 to 12,  
13 wherein the PUFA is an n-3 fatty acid.

14

15 14 A method as claimed in any of Claims 9 to 13,  
16 wherein the semen with the added seminal fluid is then  
17 placed in cryoscopic storage.

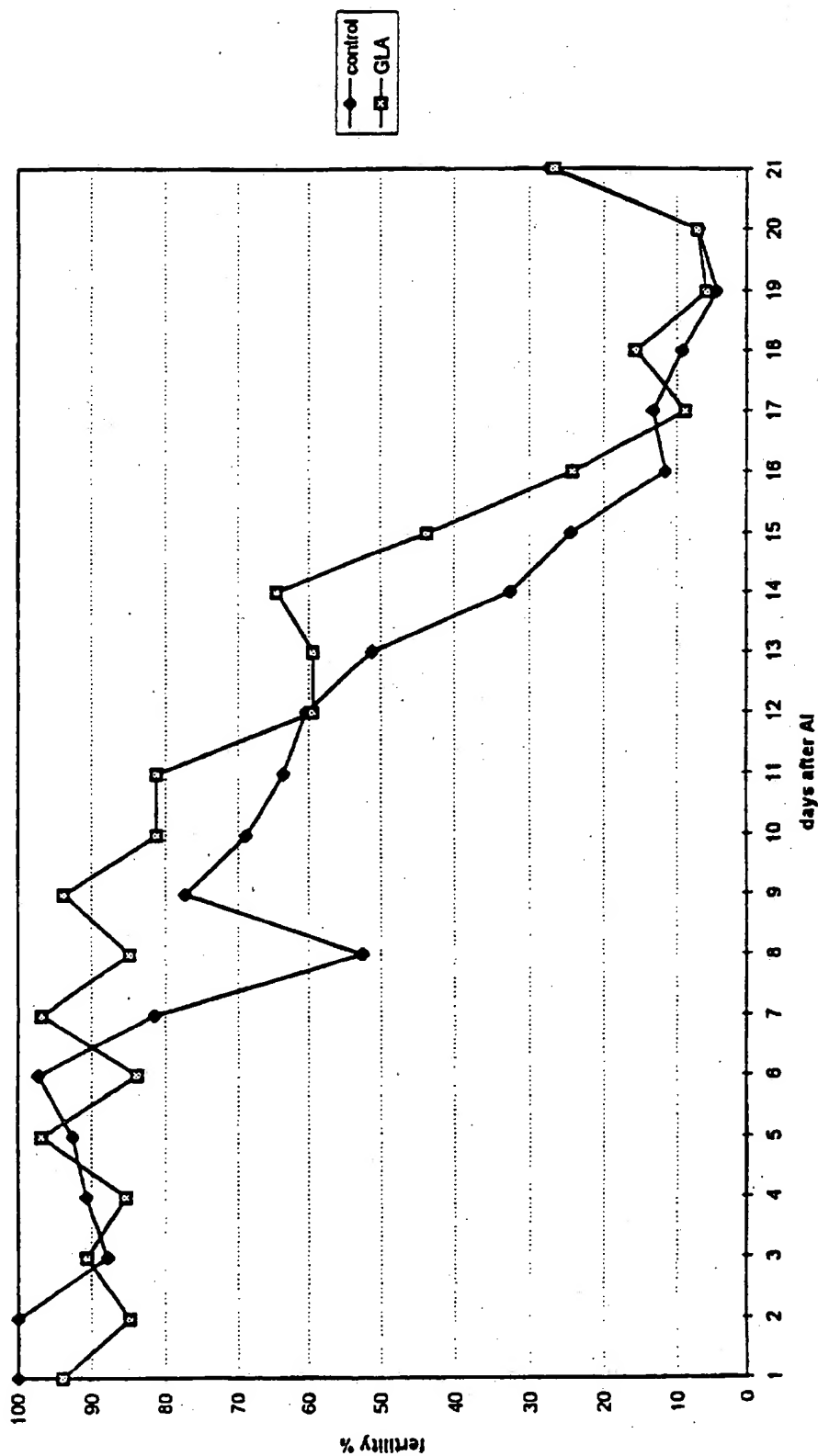
18

19 15 A method as claimed in any of Claims 9 to 14,  
20 wherein the semen with the added seminal fluid is used  
21 for artificial insemination.

22

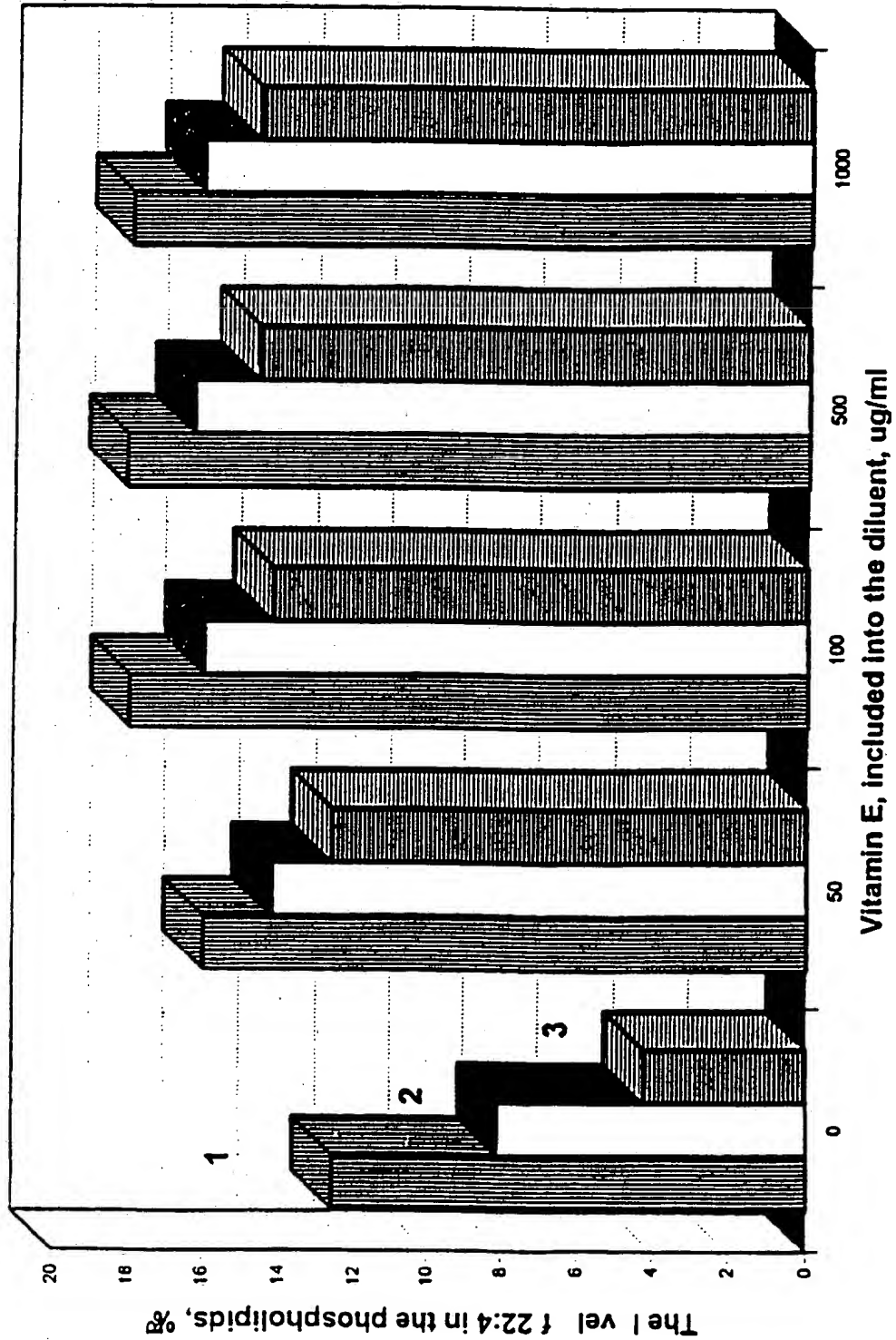


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Figure 1: Fertility rate on daily basis after 1 single AI with a dose of  $10 \times 10^7$  sperm

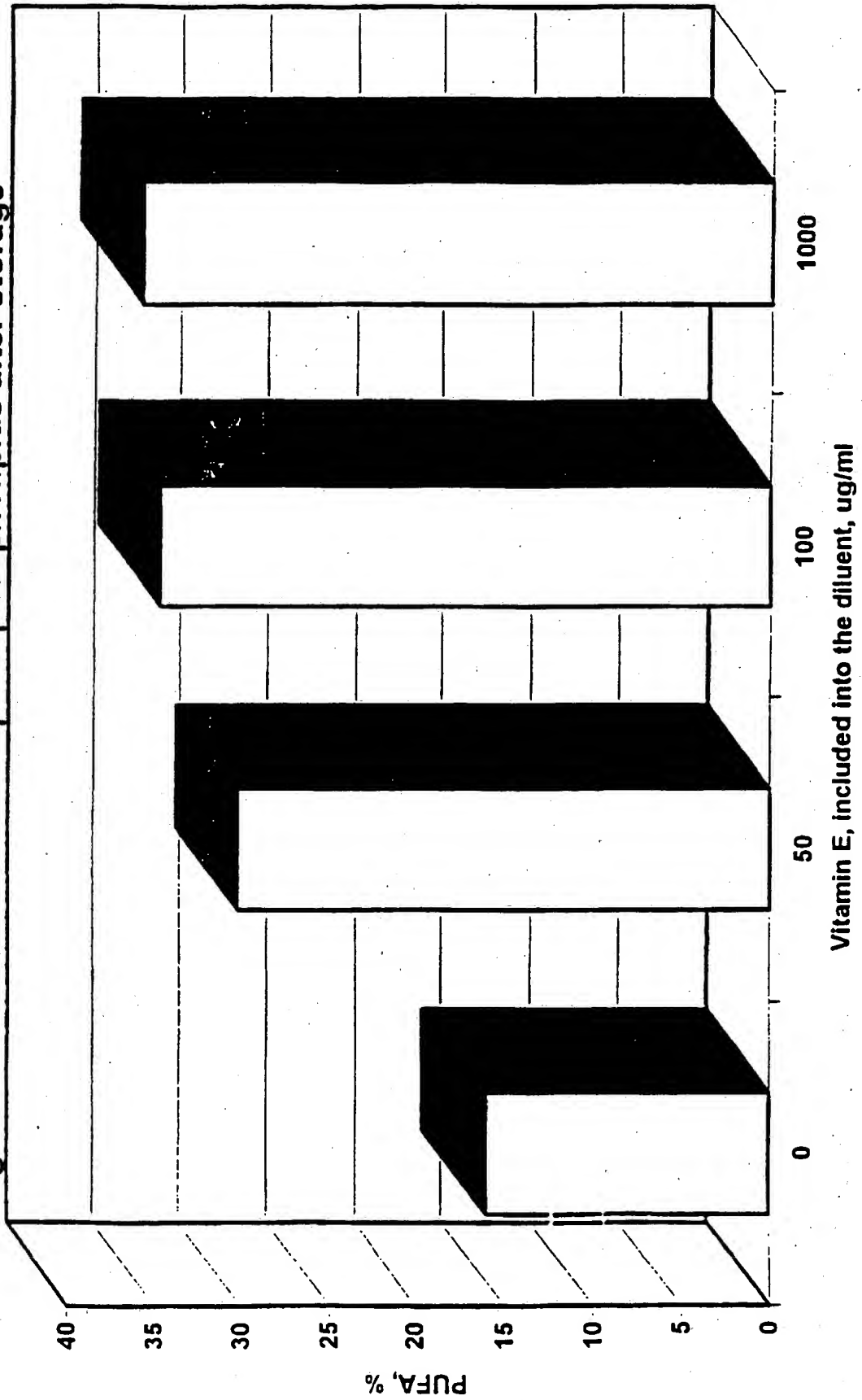
2/12

Figure 2. The levels of 22:4 fatty acid in the sperm phospholipids after storage during 24 (1), 48 (2) or 72 (3) hours at 4 C



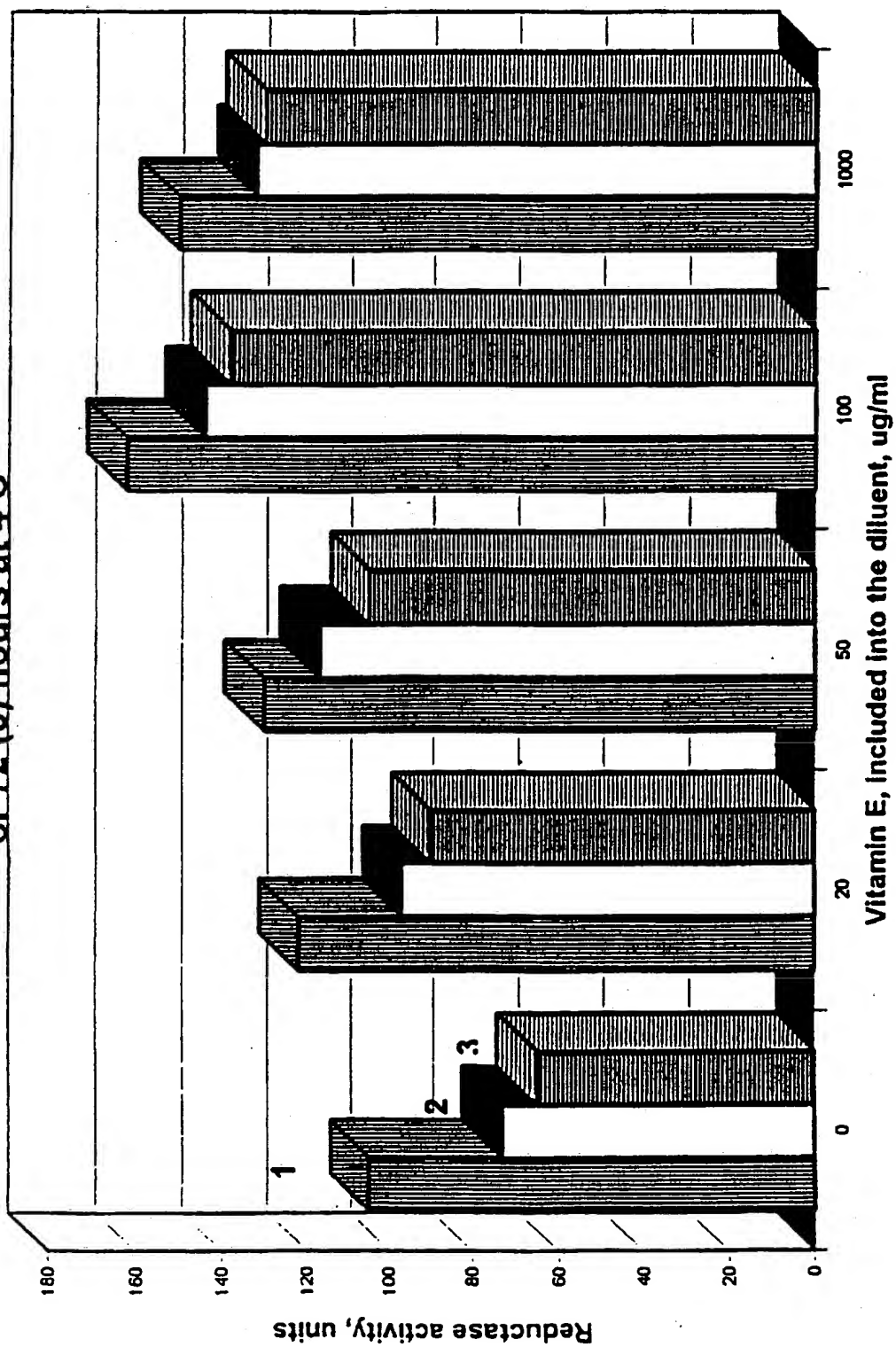
3/12

Figure 3. PUFA levels in the sperm phospholipids after storage



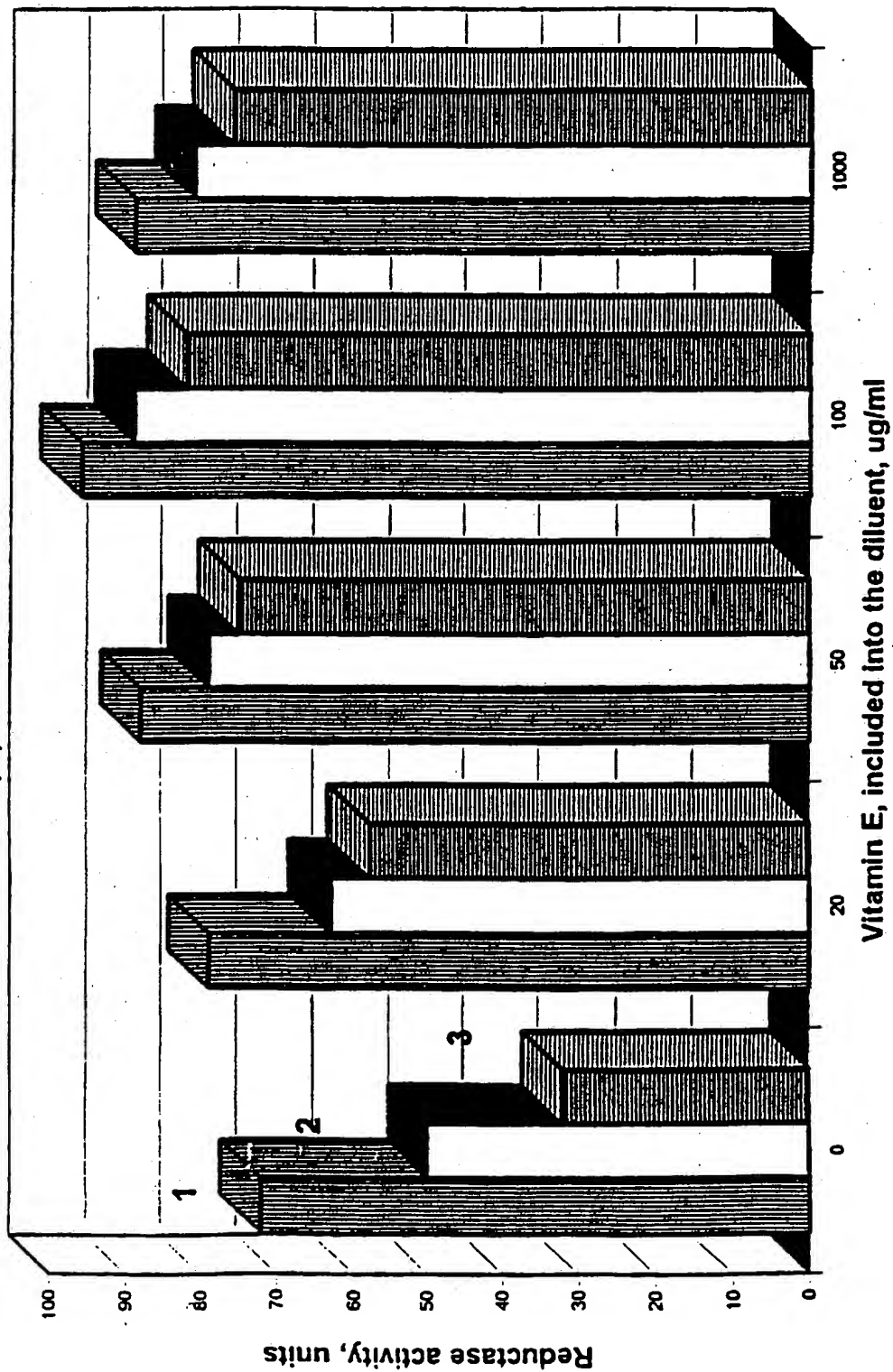
4/12

Figure 4. Reductase activity in the sperm after storage during 24 (1), 48 (2) or 72 (3) hours at 4 C



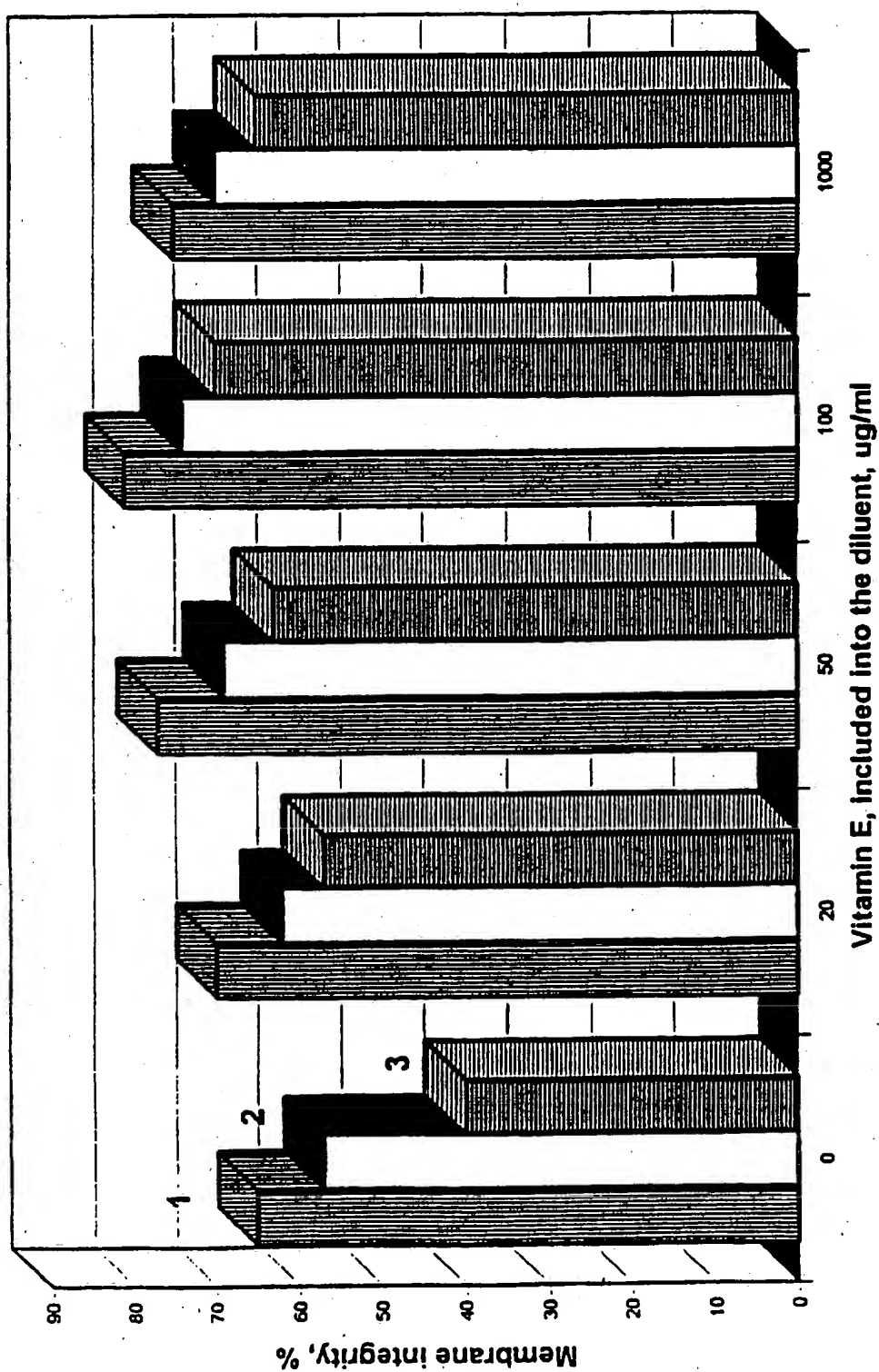
5/12

Figure 5. Reductase activity in the sperm after storage during 6 (1), 12 (2) or 24 (3) hours at 37 C



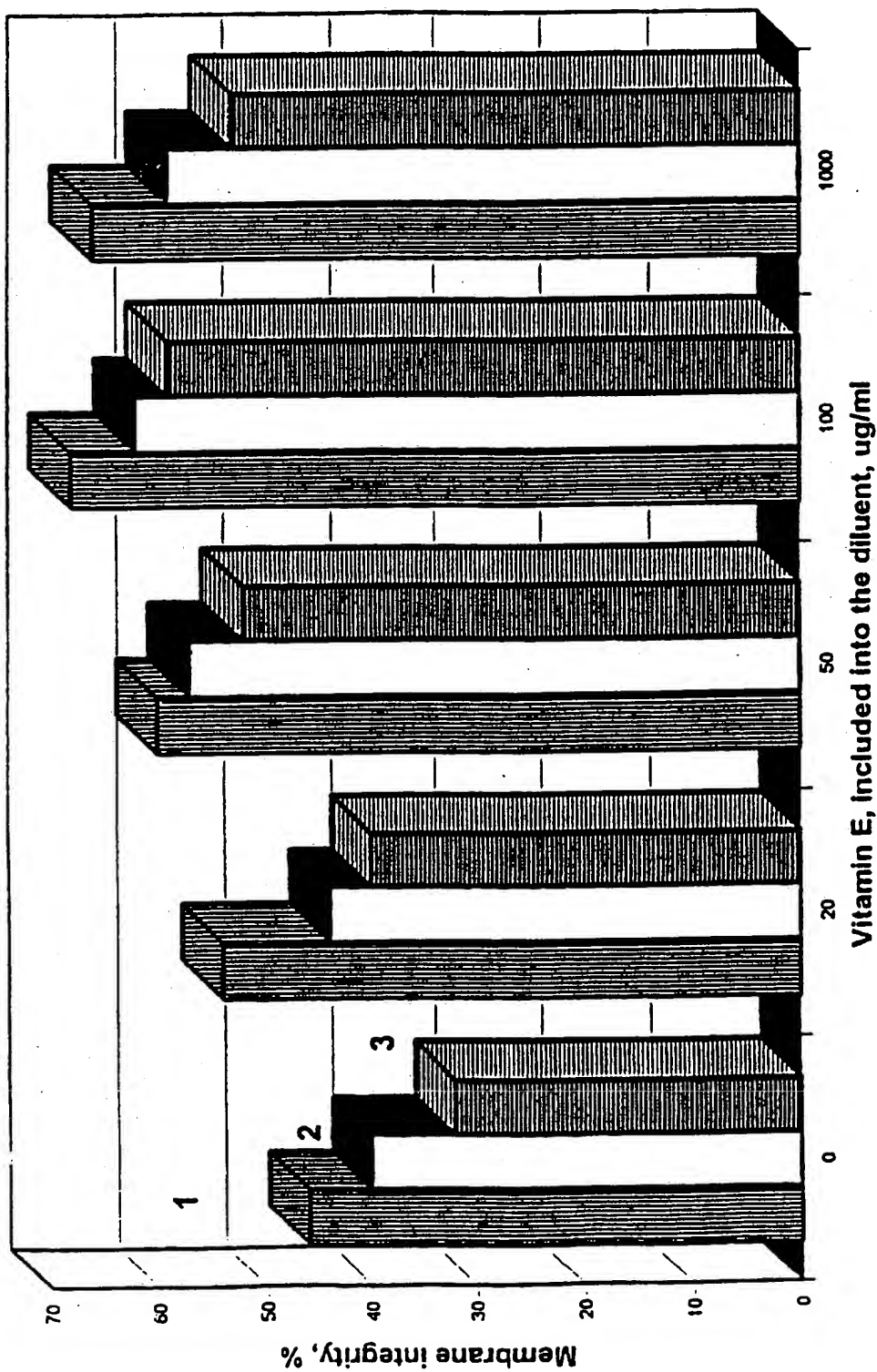
6/12

Figure 6. Sperm viability after storage during 24 (1), 48 (2) or 72 (3) hours at 4 C



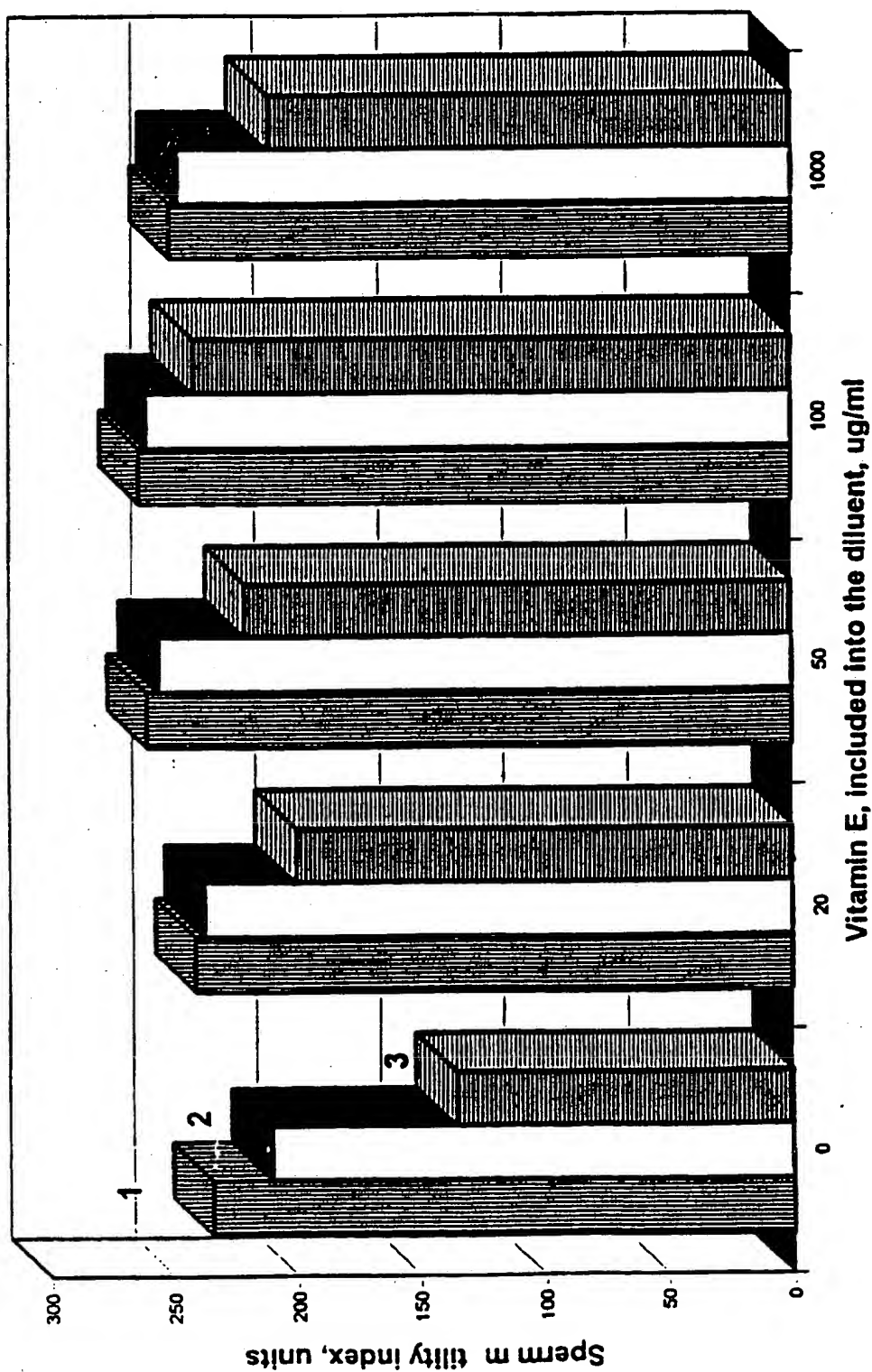
7/12

Figure 7. Sperm viability after storage during 6 (1), 12 (2) or 24 hours at 37 C



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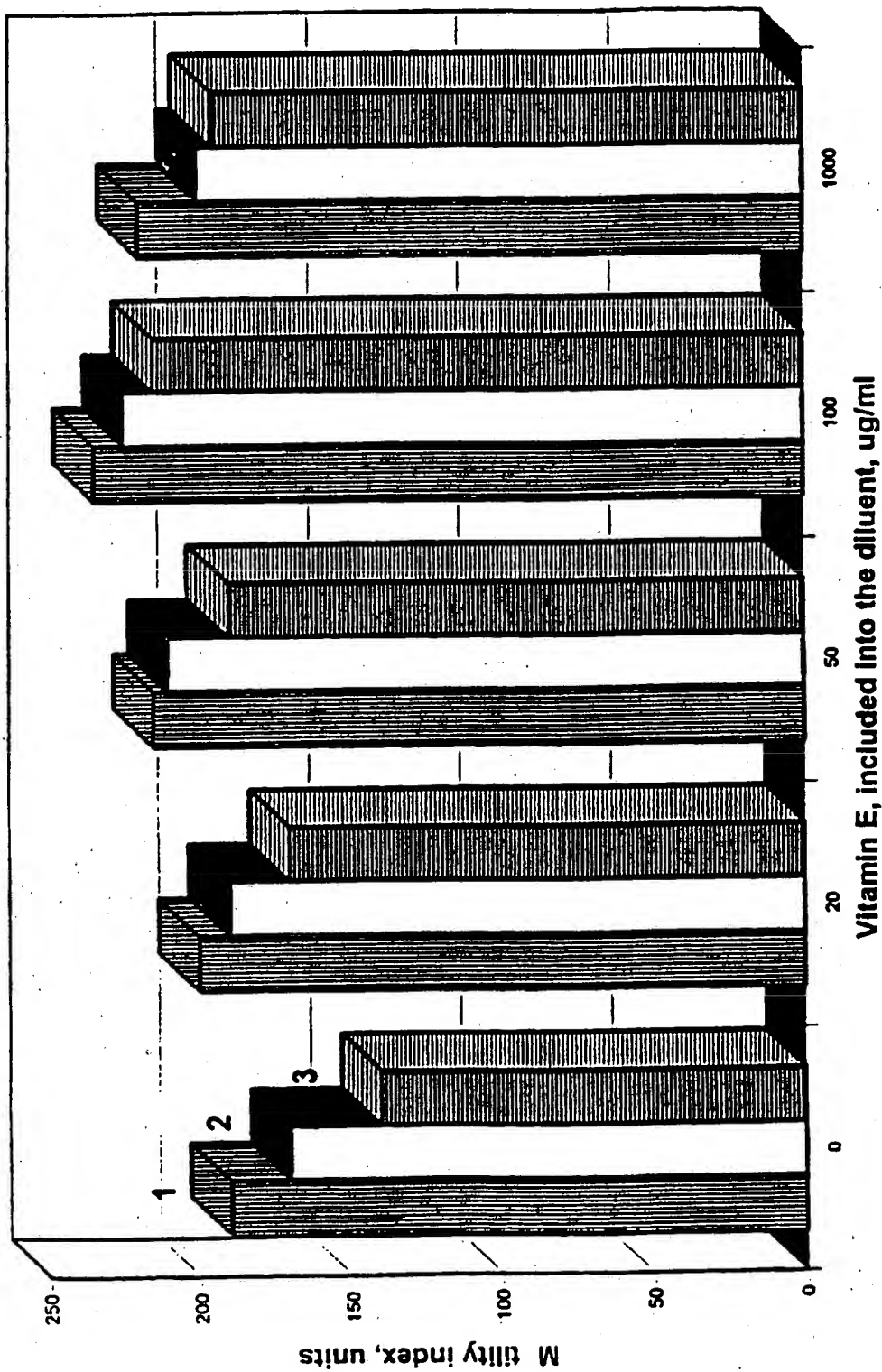
Figure 8. Sperm motility after storage during 24 (1), 48 (2) or 72 (3) hours at 4 C





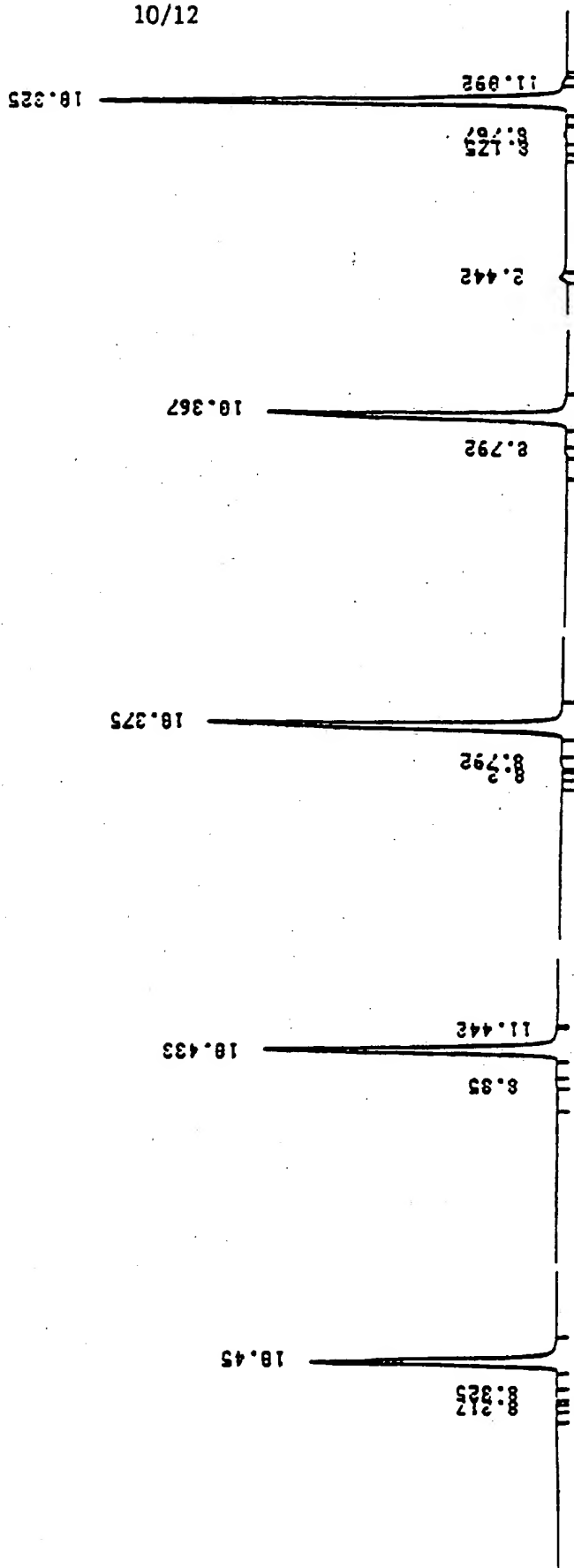
9/12

Figure 9. Sperm motility after storage during 6 (1), 12 (2) or 24 (3) hours at 37 C



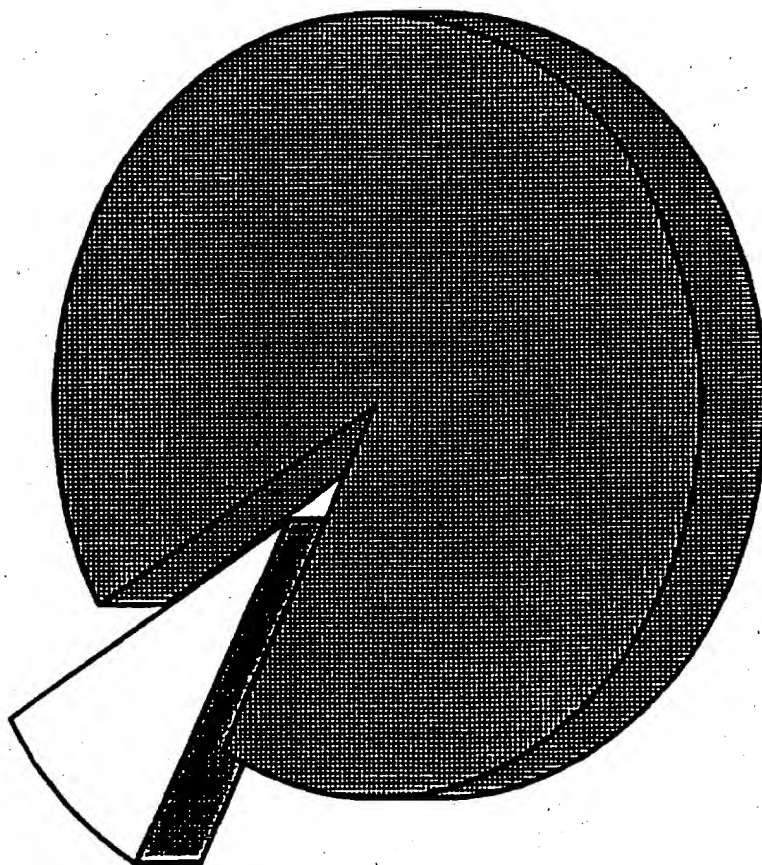
10/12

**Figure 10. Alpha-tocopherol distribution in the seminal  
plasma enriched by vitamin E**  
(400µg/ml, 4 replicates and standard solution)



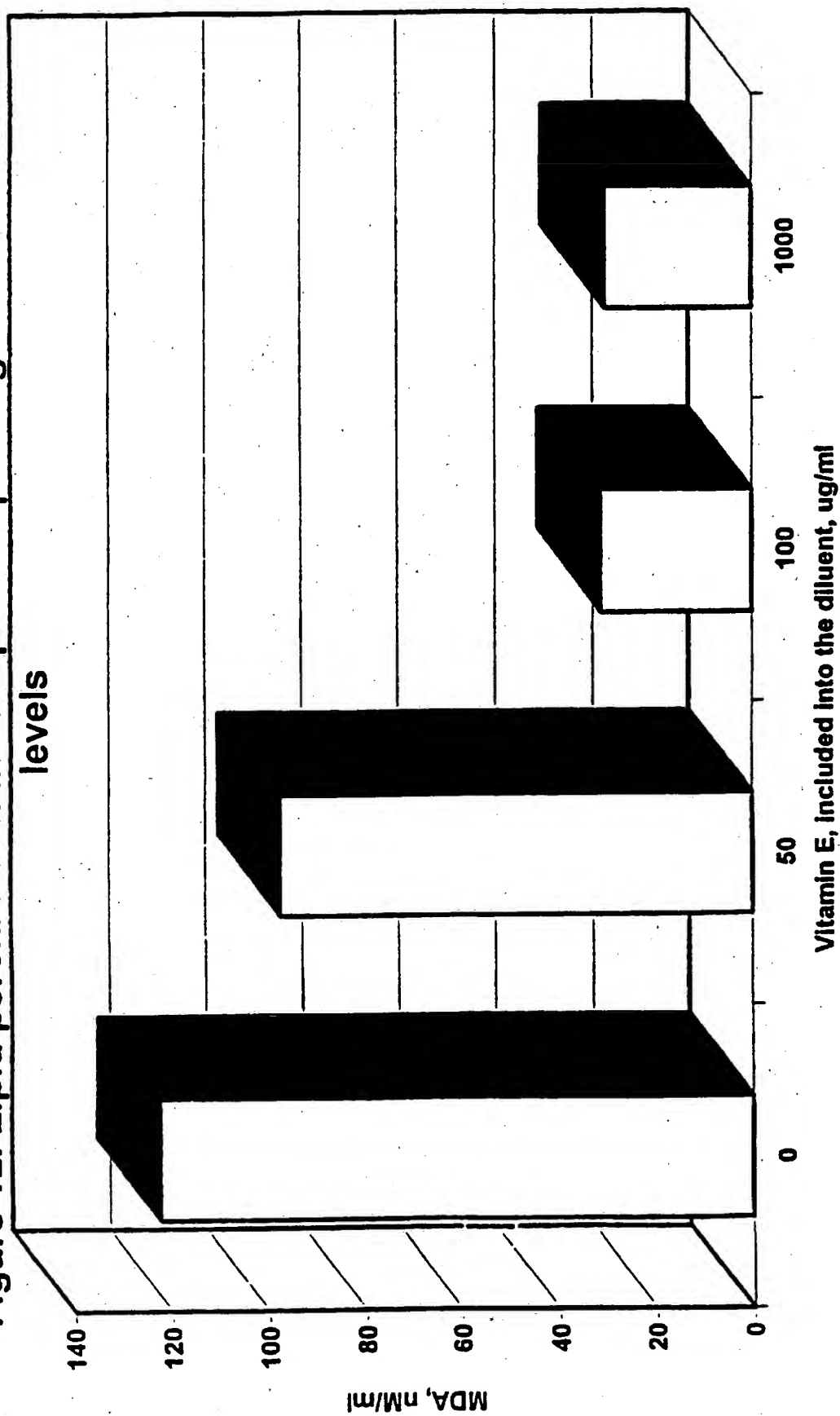
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**Figure 11. Vitamin E incorporation into the spermatozoa**



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Figure 12. Lipid peroxidation in the sperm depending on vitamin E levels



# INTERNATIONAL SEARCH REPORT

national Application No  
PCT/GB 97/01735

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61K31/20 A61K31/335

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	H.M. SINCLAIR: "Essential fatty acids in perspective." HUM. NUTR. CLIN. NUTRITION., vol. 38, no. 4, 1984, pages 245-260, XP002044092 see page 251	1
X	H. PAULENZ ET AL.: "A preliminary study on the effect of dietary supplementation with cod liver oil on the polyunsaturated fatty acid composition of boar semen." VET. RES. COMMUN., vol. 19, no. 4, 1995, pages 273-284, XP002044093 * discussion *	2,4,6
A	---	9-15
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

21 October 1997

Date of mailing of the international search report

31. 10. 97

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## INTERNATIONAL SEARCH REPORT

National Application No

PCT/GB 97/01735

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	E. KESSOPOULOU ET AL.: "A double-blind randomized placebo cross-over controlled trial using the antioxidant vitamin E to treat reactive oxygen species associated with male infertility." FERTIL. STERIL., vol. 64, no. 5, 1995, pages 825-831, XP002044094 * abstract *	1,4,7
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P,X	E. GEVA ET AL.: "The effect of antioxidant treatment on human spermatozoa and fertilization rate in an in vitro fertilization program." FERTIL. STERIL., vol. 66, no. 3, 1996, pages 430-434, XP002044095 see the whole document	1,4,7
P,X	---	
P,X	R.K. SHARMA ET AL.: "Role of reactive oxygen species in male infertility." UROLOGY, vol. 48, no. 6, 1996, pages 835-850, XP002044096	1,7
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P,X	J.E. AURICH ET AL.: "Effects of antioxidants on motility and membrane integrity of chilled-stored stallion semen." THERIOGENOLOGY, vol. 48, no. 2, 1997, pages 185-192, XP002044097 see the whole document	1,5,7
P,X	---	
P,X	WO 97 16965 A (MEDICAL RESEARCH COUNCIL.) 15 May 1997 see the whole document -----	1,5,7

**Information on patent family members**

**PCT/GB 97/01735**

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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